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Pressure-Temperature Interactions on M_4-Lactate Dehydrogenases From Hydrothermal Vent Fishes: Evidence for Adaptation to Elevated Temperatures by the Zoarcid *Thermarces andersoni*, but not by the Bythitid, *Bythites hollisi*

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Abstract. Lactate dehydrogenases (LDH; M_4 isozyme) were purified from skeletal muscle taken from two fishes endemic to hydrothermal vents, *Thermarces andersoni* (Zoarcidae; 13°N, East Pacific Rise, depth ~ 2600 m) and *Bythites hollisi* (Bythitidae; Galapagos Spreading Center, depth ~ 2500 m), and from the cosmopolitan deep-sea rattail *Coryphaenoides armatus* (Macrouridae; depth of occurrence to ~5000 m). The effects of pressure and temperature on the apparent Michaelis-Menten constant (K_m) of cofactor (NADH) were measured to compare sensitivities to temperature, at *in situ* pressures, of enzymes from hydrothermal vent fishes and from a species adapted to cold, stable deep-sea temperatures. At 5°C, the K_m of NADH of the M_4-LDHs of the three species varied only slightly between measurement pressures of 1 and 340 atmospheres (atm), in agreement with earlier studies of M_4-LDHs of deep-sea fishes. At higher measurement temperatures, marked differences were found among the enzymes. For the M_4-LDHs of *C. armatus* and *B. hollisi*, increases in temperature (10 to 20°C), at *in situ* pressures, sharply increased the K_m of NADH to values higher than those predicted to be physiologically optimal. The M_4-LDH of *T. andersoni* exhibited only minimal perturbation by elevated temperature under *in situ* pressures. The different temperature-pressure responses of these LDHs suggest that enzymes of deep-sea fishes not endemic to hydrothermal vents are not adapted for function at the higher temperatures found at vent sites, and that *T. andersoni* is better adapted than *B. hollisi* for sustained exposure to warm vent waters. The importance of adaptation to warm temperatures in the colonization of vent habitats is discussed.

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Introduction

The hydrothermal vent sites at seafloor spreading centers in the Eastern Pacific are, in several ways, unusual deep-sea environments: the food-chain is based on bacterial chemosynthesis rather than photosynthesis; a high degree of endemism characterizes the fauna (Newman, 1985); animal biomass is enormous; and water temperatures are much higher than is typical of the deep sea (~2–3°C) (Hessler and Smithey, 1984; Grassle, 1985). The primary focus of physiological and biochemical research with vent organisms has been on the chemosynthetic processes supporting the food web, and on the adaptations of vent animals to withstand hydrogen sulfide, the primary energy source for chemosynthesis (Grassle, 1985; Somero *et al.*, 1989). Less attention has been paid to the potential importance of temperature as a factor influencing the physiologies of the vent organisms and effecting the distribution of endemic vent species and other deep-sea animals in and near the vent fields.

Temperature typically is a major influence on organismal distribution patterns and physiological function (Hochachka and Somero, 1984; Cossins and Bowler,

1987), and the steep temperature gradients found at the hydrothermal vents—up to ~380°C over distances of several cm (Fustec *et al.*, 1987)—could present challenging thermal adaptation problems to the vent fauna. Many vent invertebrates encounter temperatures considerably higher than those experienced by deep-sea species living outside the vents. Sessile invertebrates, in particular, live continuously in the warm vent effluents in which temperature can vary between about 2 and 15°C at the Galapagos Spreading Center sites (Hessler and Smithey, 1984; Johnson *et al.*, 1988), and between 2 and at least 20°C at the 13°N site on the East Pacific Rise (EPR) (Fustec *et al.*, 1987). The motile brachyuran crab *Bythograea thermydron* also forages for extended periods in the warm vent waters, and this species appears well adapted for function under conditions of high pressure and elevated temperatures (Arp and Childress, 1981; Mickel and Childress, 1982a, b). Adaptations of hydrothermal vent fishes to high temperature and pressure have not previously been investigated. Although about 20 species of fishes have been described in the general area of the vents (Cohen and Haedrich, 1983), only three fishes, all endemic species, occur within the vent field, and are potentially exposed to waters with elevated temperatures. Two are zoarcids: *Thermarces cerberus* has been identified at both the Galapagos and 21°N site on the EPR; and *T. andersoni* is found at the 13°N EPR site (Rosenblatt and Cohen, 1986). Geistdoerfer (1985), however, regards these two zoarcids as one species.

The hydrothermal vent zoarcids have been observed resting on the basaltic seafloor and, at EPR sites, on the rough surfaces of "smoker" chimneys. EPR sites are characterized by these chimneys which emit hot (up to ~380°C; black smokers) and warmed (~20°C; white smokers) waters (Hekinian *et al.*, 1983). At the EPR sites, cooler water is emitted from fissures in the seafloor. Each vent type—the hot black smokers, the white smokers, and the warm seeps from fissures—has a distinct faunal assemblage associated with it. At all three vent types, *Thermarces* are found in close association with the benthic invertebrates (Fustec *et al.*, 1987). The exact water temperatures encountered by the zoarcids are not known. But, because they have been observed to rest motionless on the bottom among the vestimentiferan tube worms and other invertebrates that live in the warm vent effluents, they may experience warm temperatures for periods long enough to effect thermal equilibration of their bodies with the warm vent waters (Fustec *et al.*, 1987).

The third vent fish described, *Bythites hollisi* (family Bythitidae) (Cohen *et al.*, 1990), has been collected only at the Galapagos Spreading Center, although fishes of similar appearance have been observed from submersibles on the EPR. *B. hollisi* is the only endemic vertebrate common to the Galapagos site (Hessler and Smithey, 1984). Individuals have been observed hovering over warm water vent openings, sometimes with their heads protruding into the cracks from which the warm water is seeping. Given this behavior, *B. hollisi* probably is exposed to water temperatures warmer than ambient deep-sea temperatures. However, the extreme steepness of the thermal gradients above the Galapagos-type warm water vents (up to ~13°C differences over a few cm; see Hessler and Smithey, 1984; Johnson *et al.*, 1988) precludes accurate estimates of the temperatures encountered by *B. hollisi*. Smoker chimneys are absent at the Galapagos site, so there is no potential for *B. hollisi* of this vent habitat to encounter the high temperatures that might confront fishes inhabiting the EPR sites.

A number of fishes typical of the cold deep sea, including rattail fishes (Macrouridae), have been observed swimming near the Galapagos and EPR vent sites (Cohen and Haedrich, 1983). The cosmopolitan rattail *Coryphaenoides armatus* is likely to be found at the depths of the Galapagos Spreading Center and at the 13°N and 21°N EPR sites.

M_4-LDHs have been studied extensively in shallow- and deep-living fishes (Siebenaller, 1987; Siebenaller and Somero, 1978, 1979, 1989), but only at a measurement temperature of 5°C. At this low temperature, the M_4-LDHs of adult fishes occurring at depths greater than 500–1000 m (51–101 atm pressure), differ adaptively from the M_4-LDH homologs of shallow-living, cold-adapted fishes. For example, the effects of pressure on the apparent Michaelis-Menten constant (K_m) of cofactor (NADH) are small or non-existent for the M_4-LDHs of deep-sea species, but very large in the case of the M_4-LDHs of shallow-living fishes. These sharp differences in the effect of pressure on the K_m of cofactor and substrates for LDHs and other enzymes (Siebenaller and Somero, 1989) are hypothesized to play important roles in establishing the depth distribution patterns of marine fishes. Analogously, differences among deep-sea species in the effects of temperature on their enzymes under *in situ* pressures might play a role in determining horizontal distribution patterns related to temperature gradients near hydrothermal vent sites.

To determine whether differences in temperature adaptation exist between the biochemistries of endemic vent fishes and deep-sea fishes from cold, thermally stable waters, we studied the skeletal muscle isozymes ($M_4 = A_4$) of lactate dehydrogenase (LDH; EC 1.1.1.27); the kinetic and structural properties of this enzyme strongly reflect the temperatures and pressures to which an organism is adapted (Yancey and Somero, 1978; Siebenaller and Somero, 1989). M_4-LDHs from *T. andersoni*, *B. hol-*

lisi, and *C. armatus* were purified and studied kinetically over a range of pressures and temperatures to determine how temperatures typical of warm water vents affect the response of M_4-LDHs to *in situ* pressures.

Materials and Methods

Collection and preservation of specimens

The specimen of *B. hollisi* (initial description by Cohen *et al.*, 1990) was captured by net from the DSV *Alvin* at the Galapagos Spreading Center during the Galapagos-1988 expedition. The specimen was returned to the surface in an insulated container and immediately dissected. Muscle samples were frozen immediately in liquid nitrogen, and returned to the Scripps Institution of Oceanography (SIO) for analysis.

The specimen of *T. andersoni* was captured in a baited trap at the 13°N EPR site during the autumn 1987 French-US Hydronaut expedition. Recovery was achieved using the French submersible DSV *Nautile*. The specimen was frozen immediately upon return to the ship, returned to SIO, and stored at −80°C until analyzed.

C. armatus was collected by otter trawl in Monterey Canyon at a depth of ~3000 m. White muscle was dissected from the fish, wrapped in aluminum foil, and frozen immediately on dry ice. Tissues were returned to SIO and stored at −80° until analyzed.

Enzyme purification and determinations of K_m of NADH

The M_4 isozyme of LDH was purified with an oxamate affinity column, as described by Yancey and Somero (1978). Native starch and polyacrylamide gels stained for LDH activity revealed a single band of activity, the M_4-LDH. SDS-polyacrylamide gels stained with Coomossie blue showed a single protein band corresponding in M_r to LDH.

The K_m of NADH was determined using an 80 m*M* imidazole/Cl buffer (pH 7.0 at 20°C). This buffer was chosen, rather than the Tris/Cl buffer used in earlier studies of the effects of pressure on LDH (*cf.* Siebenaller and Somero, 1978, 1979), because the pK of imidazole varies with temperature in parallel with the intracellular pH (pH_i) of fish muscle (Reeves, 1977). The pH values of imidazole/Cl buffers, like those of Tris/Cl buffers, are virtually unaffected by pressures in the range used in these studies (Kauzmann *et al.*, 1962). Except for the differences in assay medium (buffer species and KCl concentration; *cf.* Siebenaller and Somero, 1978), the high pressure assays were made following the protocol of Siebenaller and Somero (1978). Seven to nine concentrations of NADH spanning the value of K_m were used to determine each K_m value. The K_m values were computed according to the weighted linear regression method of Wilkinson (1961) (Wilman4 software; Brooks and Suelter, 1986). Standard deviations of the K_m values did not exceed 12% of the K_m values (Fig. 1).

Results

The effects of temperature on the pressure sensitivities of the K_m of NADH for the M_4-LDHs of the three species are illustrated in Figure 1. At 5°C, the kinetics of these enzymes resembled those of the high pressure-adapted M_4-LDHs of other deep-sea fishes (see Siebenaller, 1987; Siebenaller and Somero, 1989). Increased pressure caused at most a slight increase in the K_m of NADH, and this increase occurred over the first 68 atm rise in measurement pressure. Pressures above 68 atm caused no further increase in K_m.

At temperatures above 5°C, the M_4-LDH of *T. andersoni* differed from the homologs of the other two deep-sea species (Figs. 1, 2). At *in situ* pressures (~250 atm; dashed vertical line in Fig. 1), the M_4-LDH of *T. andersoni* exhibited no increase in K_m of NADH between 5 and 10°C, and only a slight increase between 10 and 20°C. The M_4-LDHs of *C. armatus* and *B. hollisi* exhibited an approximate doubling of the K_m of NADH as the temperature increased to 15 or 20°C.

Discussion

The K_m of substrate or cofactor for a given type of enzyme is strongly conserved among species at their physiological temperatures (Yancey and Siebenaller, 1987; Yancey and Somero, 1978) and pressures (Siebenaller, 1984, 1987; Siebenaller and Somero, 1978, 1989). The K_m of NADH for M_4-LDH varies at most by about 10 μM, both among species at their physiological temperatures and pressures, and across a single species' normal range of body temperatures and pressures. At temperatures or pressures above the normal physiological range, the K_m of NADH typically exhibits a large temperature- or pressure-related increase, and reaches values that no longer lie within the conserved range that is viewed as physiologically optimal. Similar trends have been seen for several enzymes, which emphasizes that enzymatic kinetic properties must be maintained within narrow ranges that are optimal for catalysis and regulation (reviewed by Hochachka and Somero, 1984; Siebenaller and Somero, 1989). Conservation of K_m and other kinetic parameters may only be observed when comparative studies of enzyme homologs are all performed in the same *in vitro* milieu; differences in ionic strength, for example, can affect the absolute values of K_m (*cf.* Siebe-

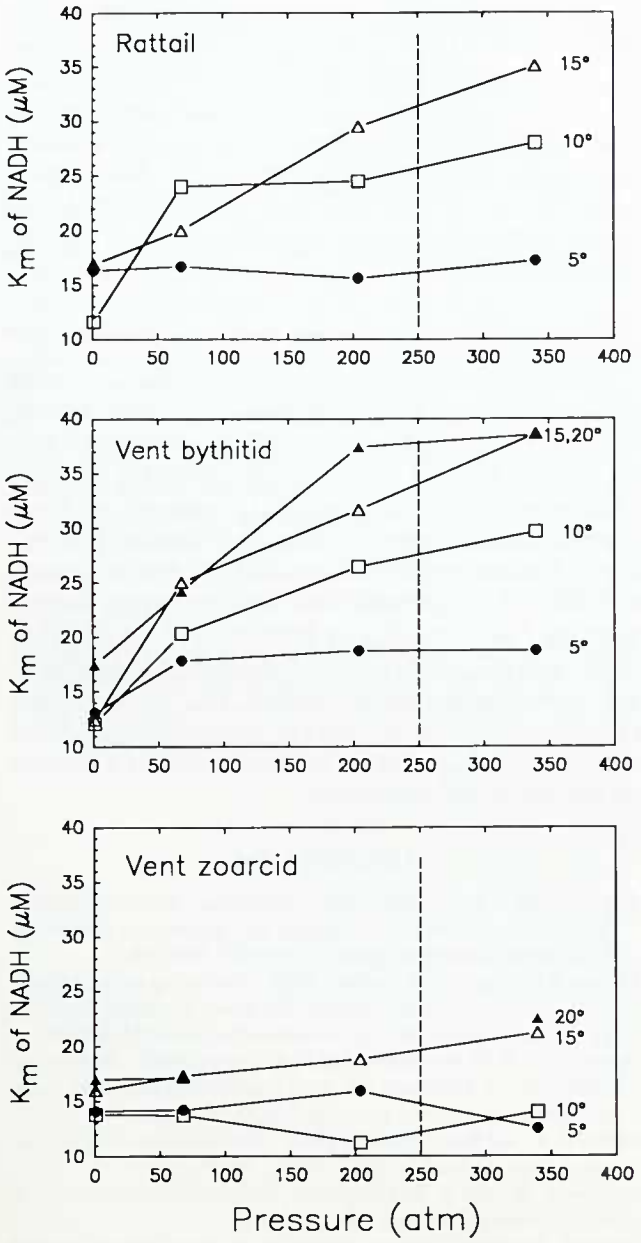

Figure 1. The effects of measurement temperature and pressure on the apparent Michaelis-Menten constant (K_m) of NADH for M_4-LDHs of the cosmopolitan deep-sea rattail fish *Coryphaenoides armatus*, the hydrothermal vent bythitid *Bythites hollisi*, and the hydrothermal vent zoarcid *Thermarces andersoni*. The dashed vertical line indicates the approximate habitat pressure at the two vent sites.

naller and Somero, 1978, with Yancey and Siebenaller, 1987).

For the M_4-LDHs of *C. armatus* and *B. hollisi*, temperatures of 10 to 20°C increased the K_m of NADH by ~15–20 μM at *in situ* pressures (Fig. 2). In contrast, the K_m of NADH for the M_4-LDH of *T. andersoni* increased by only approximately 8 μM as temperature increased from 5 to 20°C. Therefore, temperatures characteristic of warm water vents perturbed the K_m of NADH of the M_4-LDHs of *C. armatus* and *B. hollisi* sufficiently to increase their values beyond the physiologically conserved range noted for other species. The M_4-LDH of *T. andersoni* retained its K_m of NADH within the physiologically conserved range across the span of measurement temperatures at *in situ* pressure.

The different responses of the M_4-LDHs of these three species to changes in temperature at *in situ* pressure lead us to propose two hypotheses concerning the relationship between species distribution patterns and temperature and pressure influences on enzymatic function. First, we propose that the M_4-LDHs of cold-adapted deep-sea fishes are not pre-adapted for function at the elevated temperatures found at the warm water vents. Thermal perturbation of the kinetic properties of enzymes under pressure may restrict the endemic fauna of the cold deep sea from exploiting hydrothermal vent habitats. Thus, as much as interspecific differences in the pressure sensitivities of enzymes may be important in establishing species' vertical distribution patterns in the marine water column (Siebenaller and Somero, 1989), interspecific differences in the responses of enzymes to elevated temperatures, at deep-sea pressures, may be instrumental in establishing horizontal distribution patterns in temperature gradients near the deep-sea hydrothermal vents. This conjecture is not meant to imply that temperature is the only factor restricting typical deep-sea animals from the vent environment. Mechanisms for overcoming the toxic effects of hydrogen sulfide also ap-

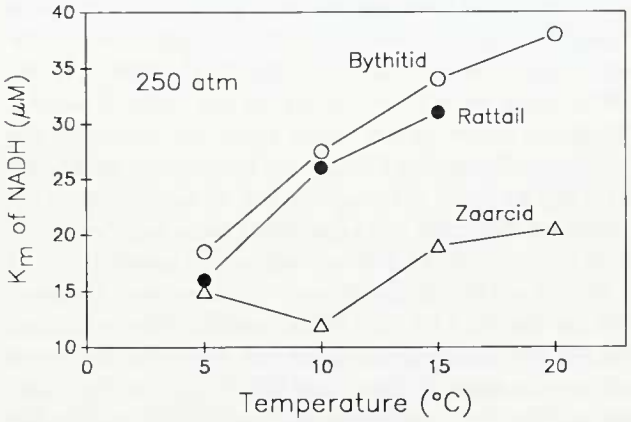

Figure 2. The effect of measurement temperature, at the approximate habitat pressure of the two hydrothermal vent sites (~250 atm), on the K_m of NADH for the M_4-LDHs of the three species shown in Figure 1. K_m values at 250 atm were estimated by the intersection of the vertical dashed line (corresponding to 250 atm pressure) with the lines connecting the K_m values at each temperature (see Fig. 1).

pear to be important components of adaptation to the vent environment (Somero *et al.*, 1989).

Second, we hypothesize that, among endemic vent species, there may be substantial differences in tolerance of high temperature and, therefore, in the microhabitats they experience. The interacting effects of elevated temperature and pressure on its M_4-LDH suggest that *B. hollisi* from the Galapagos Spreading Center is not adapted for continuous existence in the warmest waters found at this site. In contrast, by our enzymatic criterion, *T. andersoni* appears well adapted to body temperatures as high as 20°C.

Because the exact temperatures experienced by endemic vent fishes, and the times over which they remain in warm waters, are not known with accuracy, links between enzymatic properties and environmental distributions remain speculative. However, the contrasting thermal properties of their environments suggest that the two vent fishes used in these studies have different thermal experiences. At the Galapagos Spreading Center site, where *B. hollisi* is the most abundant endemic vertebrate, smoker chimneys are absent, and the highest temperature recorded in the warm water vents was ~15°C (Johnson *et al.*, 1988). Although *B. hollisi* is commonly found hovering over the vent openings, and may even enter the sites of venting (Robert R. Hessler, Scripps Institution of Oceanography, pers. comm.), the extremely steep thermal gradients characteristic of the vents make precise estimates of the fish's body temperature impossible. *B. hollisi*, unlike *T. andersoni*, appears to spend most of its time swimming and, therefore, may select water temperatures that are lower than those encountered by the demersal zoarcid, which commonly rests among sessile invertebrates living directly in the warm vent effluent. At the 13°N EPR site, where *T. andersoni* is the most abundant endemic vertebrate, the temperatures of the warm water vents reach at least 20°C (Fustec *et al.*, 1987). Zoarcids are also found on the walls of smoker chimneys, where waters much hotter than those at the Galapagos Spreading Center are emitted. Zoarcids are observed to swim very rapidly out of hot smoker-vent waters, so they may not experience these high temperatures for more than a few seconds per encounter.

Recent studies of the effects of pressure and temperature on the K_ms of NADH of malate dehydrogenases (MDHs) of invertebrates from the hydrothermal vents and several other shallow- and deep-water marine habitats support the hypothesis that adaptation to elevated temperatures is important for vent species exposed to warm vent effluents for extended periods (Dahlhoff, 1989; Dahlhoff and Somero, in prep.). Although all of the MDHs from deep-sea invertebrates were found to be pressure insensitive at 5°C, only the warm-adapted hydrothermal vent species exhibited the pattern of stability of the K_m of NADH under high pressure and elevated temperature shown here for the M_4-LDH of *T. andersoni*. We propose, then, that the hydrothermal vent animals, which attain thermal equilibrium with the warm vent waters, are characterized by pervasive biochemical adaptations to elevated temperatures, and these adaptations are prerequisite to an exploitation of the warm microhabitats in the vent field.

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